Studies of extracellular fibronectin matrix formation with fluoresceinated fibronectin and fibronectin fragments

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Fluorescein isothiocyanate conjugated human plasma fibronectin, 70-kDa collagen-binding, 60-kDa central, 60-kDa heparin-binding, 180-kDa heparin, collagen-binding fibronectin fragments and gelatin were used to study extracellular fibronectin matrix formation. Exogenous fibronectin, gelatin, 70-kDa collagen-binding and 180-kDa heparin, collagen-binding fragments were shown to be able to bind specifically to preexisting extracellular matrix of living fibroblasts. The results suggest that: (i) Fibronectin matrix formation may occur through a self-assembly process; (ii) the NH_2-terminal part of fibronectin is responsible for fibronectin-fibronectin interaction during fibronectin fibril formation; (iii) plasma fibronectin may be the source for tissue fibronectin.

Fibronectin *Fibronectin fragment* *Extracellular matrix formation*

1. INTRODUCTION

The cells of eucaryotic organisms are bathed in an environment of extracellular matrices. The importance of matrices for different basic cellular functions is now generally accepted [1]. Fibronectin is a high molecular mass glycoprotein that is a major component of extracellular matrix [2–4]. In extracellular matrices, besides binding to cell surface and other matrix components such as collagen and proteoglycans, fibronectin molecules associate directly to form filaments. Antiserum raised against human plasma fibronectin has been used to localize fibronectin by immunofluorescence, which reveals a 3-dimensional matrix consisting of fine fibrils. Cellular fibronectin exists as an insoluble tissue protein found in the extracellular matrix and on cell surfaces. In a soluble form fibronectin is present in human plasma (0.3 mg/ml). The function of plasma fibronectin is not yet known. Recently, in vivo and in vitro experiments have demonstrated that plasma fibronectin can be incorporated into extracellular matrices of different tissues and cultured cells [5]. It has been suggested that plasma fibronectin may provide a reservoir for tissue fibronectin [6].

Here, the role of fibronectin-fibronectin interactions in extracellular matrix formation was investigated. We demonstrated that exogenous fibronectin, 180-kDa heparin, collagen-binding fragment, 70-kDa collagen-binding fragment and gelatin were able to bind specifically to extracellular matrix of living fibroblasts. Results obtained suggest that plasma fibronectin may be a source for tissue fibronectin and that fibronectin matrix formation may occur through a self-assembly process on preexisting fibronectin fibrils.

2. MATERIALS AND METHODS

Fibronectin was isolated from human plasma by gelatin-Sepharose chromatography [8]. Tryptic fragments of M_r = 180000 (180-kDa heparin, collagen-binding), 70000 (70-kDa collagen-binding), 60000 (60-kDa central), 60000 (60-kDa heparin-binding) were produced and isolated as

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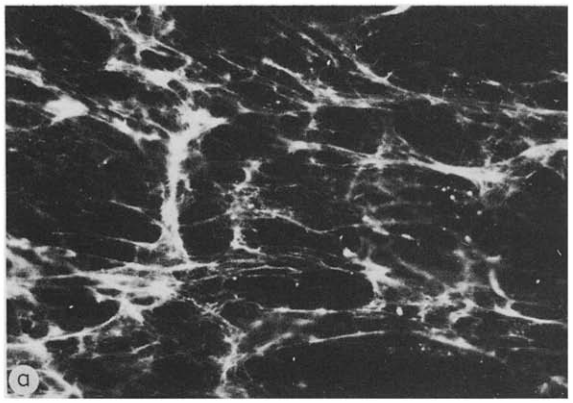
described earlier [9]. Fibronectin, its fragments, gelatin and IgG at concentrations 1–2 mg/ml were conjugated to fluorescein isothiocyanate (0.1 mg/ml) in 0.1 M carbonate-bicarbonate buffer (pH 9.3) at room temperature for 2 h with constant stirring. Unreacted dye was removed by gel filtration through a Sephadex G-25 column in phosphate-buffered saline (pH 7.4) (PBS). The ratio of fluorescein to proteins was 3–4. Fluorescein isothiocyanate-conjugated proteins were stored in aliquots at −70°C.

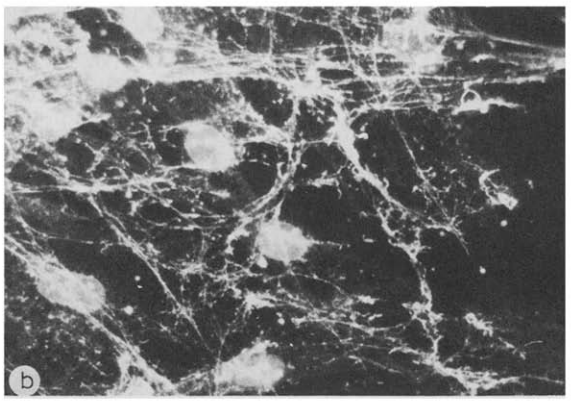
Human skin fibroblasts were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. All experiments were performed between passages 8–10 and 2 days after the cells reached confluence. Cells on coverslips were washed twice in DMEM and incubated with 0.5 mg/ml FITC-protein in DMEM at 37°C for 4 h. The coverslips were washed twice in DMEM, fixed with 5% paraformaldehyde for 20 min, rinsed, mounted in 50% glycerine-PBS and photographed on a Zeiss photomicroscope III equipped with phase contrast and epifluorescence.

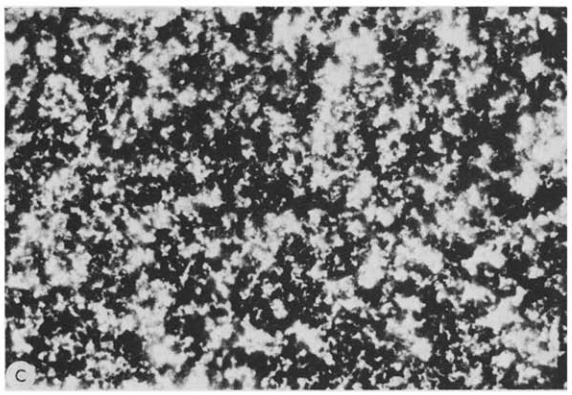
Isolated cell matrices were obtained by extracting confluent cell layers with buffer, containing 0.025 M Tris-HCl, 0.01 M EGTA, 1% Triton X-100 (pH 9.6) for 20 min at 20°C. Cell-free matrix was washed in DMEM and incubated with fluoresceinated fibronectin.

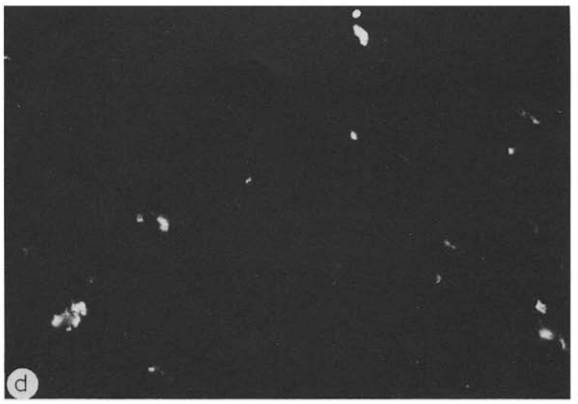
3. RESULTS AND DISCUSSION

We have used fluorescein isothiocyanate (FITC) conjugated human plasma fibronectin, fibronectin frements – 180-kDa heparin, collagen-binding, 70-kDa collagen-binding (NH_2-terminal third of the molecule), 60-kDa central, 60-kDa heparin-binding (COOH-terminal third of the molecule) – and gelatin to study the incorporation of these proteins into fibronectin matrices of living human skin fibroblasts. Fig.1a,e,f,i shows that fluoresceinated









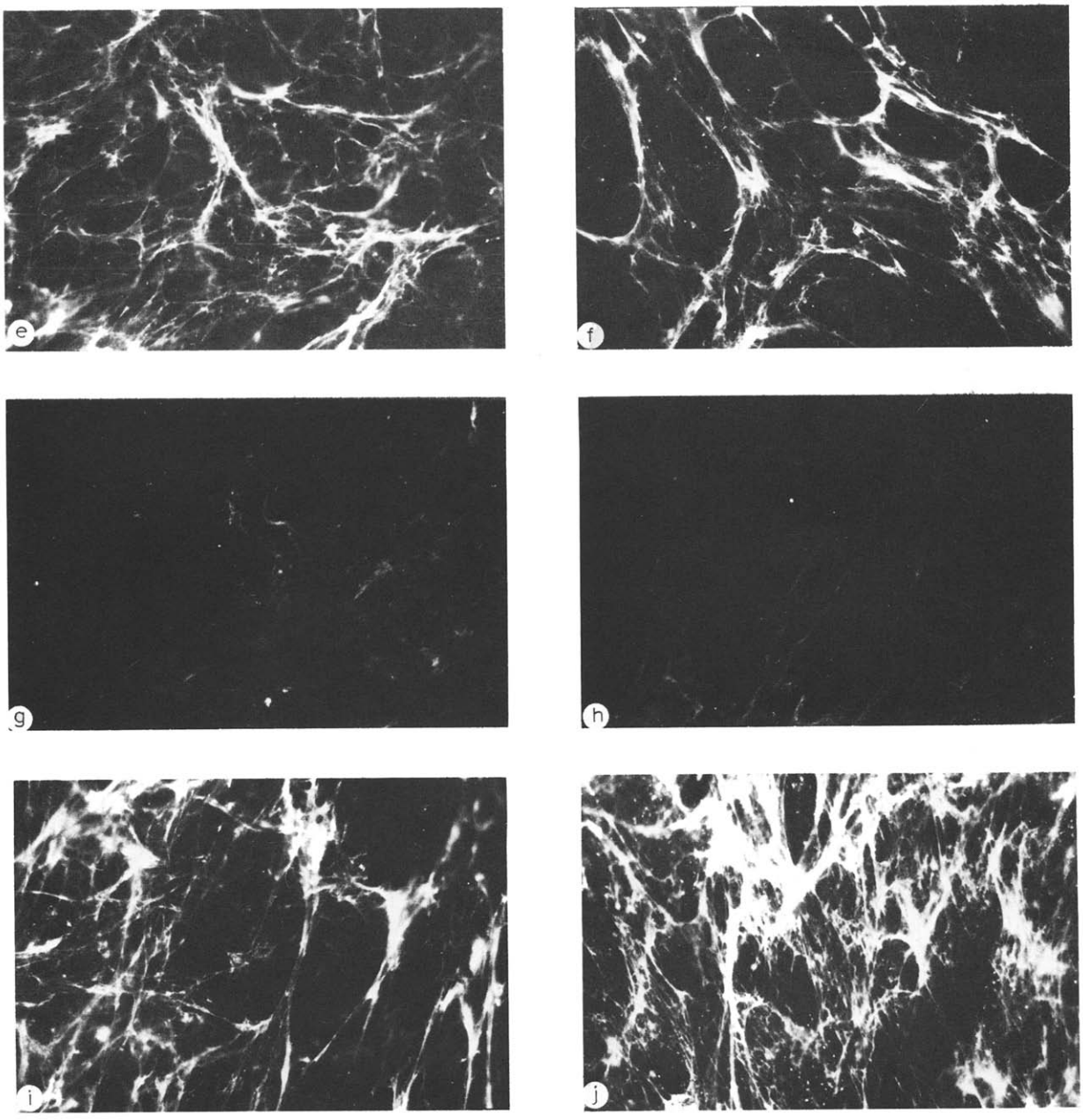


Fig.1. Binding of fluoresceinated exogenous fibronectin, fibronectin fragments and gelatin to cell layers of human skin fibroblasts. Fibroblasts were incubated with: (a) FITC-fibronectin; (d) FITC-IgG; (e) FITC-70 kDa collagen-binding fragment; (f) FITC-180 kDa heparin, collagen-binding fragment; (g) FITC-60 kDa central fragment; (h) FITC-60 kDa heparin-binding fragment; (i) FITC-gelatin. (b) Triton X-100 insoluble fibronectin matrix was incubated with FITC-fibronectin. (c) Artificial fibrillar collagen matrix was incubated with FITC-fibronectin. (j) Immunofluorescence staining of cell layer of human skin fibroblasts with antibody to fibronectin.

fibronectin, 180-kDa heparin, collagen-binding fragment, 70-kDa collagen-binding fragment and gelatin can bind to confluent cell layers of cultured human skin fibroblasts. Fluoresceinated fibronectin, some fibronectin fragments and gelatin at concentrations of 0.5 mg/ml stain an extensive network of fibers of fibroblast extracellular matrices. This fibrous network consists of fibers of different apparent diameters. Fluoresceinated IgG and some fibronectin fragments – 60-kDa central and 60-kDa heparin-binding – practically do not bind to cell layers of fibroblasts (fig.1d,g,h). We consider the binding of fluoresceinated fibronectin and 180- and 70-kDa fibronectin fragments to be specific for several reasons: (i) the dilution of fluoresceinated fibronectin and fragments with 10-fold excess of unlabeled fibronectin decreases the fluorescence of extracellular matrices (not shown); (ii) non-immune immunoglobulin G (IgG) does not stain the matrix (fig.1d); (iii) the character of staining is typical for fibronectin location as revealed by staining of extracellular matrices with specific antibody to fibronectin and fluoresceinated gelatin (fig.1i,j); (iv) fluoresceinated fibronectin and fibronectin fragments retain their biological activity – binding to heparin and gelatin, promoting adhesion of fibroblasts (not shown). The fluoresceinated proteins became associated with extracellular matrix fibrils even after 15–20 min of incubation. Fluoresceinated fibronectin can also incorporate into detergent-insoluble extracellular matrix prepared by extracting a confluent fibroblast cell layer with 1% Triton X-100 (fig.1b). More than 90% of the proteins in this matrix are fibronectin, suggesting that the matrix is made of only one component – fibronectin [10]. The distribution of fluoresceinated fibronectin in isolated fibronectin matrix is similar compared with extracellular matrix of non-extracted with Triton X-100 fibroblasts. Fluoresceinated fibronectin and 180- and 70-kDa collagen-binding fragments also are able to stain the artificial fibrillar collagen matrices (fig.1c). In this case the character of staining was completely different from staining of natural fibronectin matrices.

It has been suggested that interactions of fibronectin with other matrix components such as collagen and proteoglycans could be important for extracellular matrix formation [11–13]. The present findings demonstrate that fibronectin-fibronectin interactions are enough for fibronectin matrix formation on preexisting fibronectin fibrils. It means that formation of extracellular fibronectin fibrils may occur through a self-assembly process. The NH_2-terminal (collagen-binding) part of the fibronectin molecule is responsible for fibronectin-fibronectin interactions during fibronectin fibril formation. This conclusion is based on: (i) data presented in this paper – the 70-kDa collagen-binding fragment was shown to be able to bind specifically to fibronectin extracellular matrix; (ii) it was found that the gelatin-binding fragment could also interact with intact fibronectin, and therefore contains a site involved in fibronectin-fibronectin associations [14]. Fibronectin-fibronectin binding is suggested by the known ability of fibronectin molecules to assemble into filaments in vitro [15]. Recently it was shown that plasma fibronectin may be incorporated into extracellular matrix via specific cell-surface receptors [7,16,17]. Immunofluorescence studies of fibroblasts treated by cytochalasin B suggest that movement of receptor-attached fibronectin on the cell surface may be involved in fibronectin fibril formation [18]. Our observations demonstrate that in addition to a cell-dependent mechanism of fibronectin fibril formation, exogenous fibronectin becomes incorporated into preexisting extracellular matrix through a self-assembly process, that may provide a mechanism for recruiting plasma fibronectin into extracellular matrix.

The binding of the exogenous 180-kDa heparin, collagen-binding fragment to the extracellular matrix of fibroblasts seems to be of special interest. This fragment lacks parts of the NH_2 (27 kDa) and COOH (6 kDa) termini of the fibronectin polypeptide chains [19,20]. The 27-kDa fragment contains the site at which fibronectin is cross-linked to fibrin and collagen by blood coagulation Factor XIII [21]. The 6-kDa fragment contains interchain disulfide bonds [20]. Therefore, the 180-kDa heparin, collagen-binding fragment is a monomer and lacks the ability to be covalently cross-linked to fibrin and collagen. In contrast to intact fibronectin, the 180-kDa heparin, collagen-binding fragment has an unfolded conformation in solution [22]. Nevertheless, this fragment may bind to extracellular matrix. It means that the NH_2-terminated 27-kDa part of the

fibronectin molecule, COOH-terminal interchain disulfide bonds and compact conformation are not necessary for incorporation of fibronectin into the extracellular matrix.

REFERENCES

[1] Hay, E.D. (1981) Cell Biology of Extracellular Matrix, Plenum Press, New York.
[2] Ruoslahti, E., Engvall, E. and Hayman, E.G. (1981) Coll. Res. 1, 95–128.
[3] Hynes, R.O. and Yamada, K.M. (1982) J. Cell Biol. 95, 369–377.
[4] Yamada, K.M. (1983) Ann. Rev. Biochem. 52, 761–799.
[5] Hayman, E.G. and Ruoslahti, E. (1979) J. Cell Biol. 88, 352–357.
[6] Oh, E., Pierschbacher, M. and Ruoslahti, E. (1981) Proc. Natl. Acad. Sci. USA 78, 3218–3221.
[7] McKeown-Longo, P.J. and Mosher, D.F. (1983) J. Cell Biol. 97, 466–472.
[8] Engvall, E. and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5.
[9] Venyaminov, S.Y., Metsis, M.L., Chernousov, M.A. and Koteliansky, V.E. (1983) Eur. J. Biochem. 135, 485–489.
[10] Chen, L.B., Murray, A., Segal, R.A., Buchnell, A. and Walsh, M.L. (1978) Cell 14, 377–391.
[11] McDonald, J.A., Kelley, D.G. and Brockelmann, T.J. (1982) J. Cell Biol. 92, 485–492.
[12] Perkins, M.E., Tae, H.J. and Hynes, R.O. (1979) Cell 16, 941–952.
[13] Rennard, S.I., Wind, M.L., Hewitt, A.T. and Kleinman, H.K. (1981) Arch. Biochem. Biophys. 206, 205–212.
[14] Ehrisman, R., Roth, D.E., Eppenberger, H.M. and Turner, D.C. (1982) J. Biol. Chem. 257, 7381–7387.
[15] Vuento, M., Vartio, T., Saraste, M., Von Bensdorff, C.H. and Vaheri, A. (1980) Eur. J. Biochem. 105, 33–42.
[16] Grinnell, F. (1980) J. Cell Biol. 86, 104–112.
[17] Pena, S.D.J. and Hughes, R.C. (1978) Nature 276, 80–83.
[18] Lyubimov, A.V. and Vasiliev, J.M. (1982) Cell Biol. Int. Rep. 6, 105–112.
[19] Ruoslahti, E., Hayman, E.G., Engvall, E., Cotran, W.C. and Bulter, W.T. (1981) J. Biol. Chem. 256, 7277–7281.
[20] Petersen, T.E., Thogersen, H.C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L. and Magnusson, S. (1983) Proc. Natl. Acad. Sci. USA 80, 137–141.
[21] Mosher, D.F., Schad, P.E. and Vann, J.M. (1980) J. Biol. Chem. 255, 1181–1188.
[22] Metsis, M.L., Sepetov, N.F., Morozkin, A.D. and Koteliansky, V.E. (1985) Biochim. Biophys. Acta, in press.